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Effects of lactalbumin enzymatic hydrolysate on human squamous cell carcinoma cells-an *in vitro* study

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ABSTRACT

Objectives: Alpha-lactalbumin, the protein from human and bovine milk has been found to be promising as an alternative of anticancer agent. This study was aimed to investigate the effects of lactalbumin enzymatic hydrolysate (LAH) on cell proliferation, migration, and mRNA expression of *matrix metalloproteinase* (*MMP*) on human squamous cell carcinoma (hSCC) cell lines, *in vitro*.

Methods: Tongue (HSC-4 and 7) and pharyngeal (HN-30 and 31) hSCC cell lines were treated with a two-fold dilution of LAH (0.39–100 mg/ml). Cell viability and cell proliferation were examined by MTT assay. Colony forming unit (CFU) was assessed by crystal violet blue staining. Cell migration was investigated by scratch wound healing assay. Gene expression of metastasis-associated *MMPs* was assessed by RT-qPCR. Statistical analyses were evaluated at p value = 0.05.

Results: LAH at concentration of 50 and 100 mg/ml exhibited cytotoxicity on hSCC cells. The proliferation and CFU ability of hSCC cells were significantly attenuated after LAH treatment. The mRNA expression of *MMP2*, *MMP9*, and *MMP14* was reduced in HN-30 and HN-31 cells while expression of *MMP2* and *MMP14* was downregulated in HSC-7 cells. Only *MMP1* mRNA level was reduced in HSC-4 cells. However, cell migration of all hSCC cell lines did not alter after LAH treatment.

Conclusion: LAH treatment exhibits inhibitory effects on hSCC cell growth, proliferation and *MMPs* gene expression. Thus, LAH should be the promising alternative agent to develop the prospective anti-cancer drug.

1. Introduction

Squamous cell carcinoma (SCC) is the most common type of malignancy in the head and neck region and is currently ranked sixth among common cancers worldwide.[1] Oral squamous cell carcinoma (OSCC) can be found in several areas in the mouth, including tongue, lip, oral cavity, oropharynx, and larynx.[2] It is primarily associated with multiple risk factors such as tobacco and betel quid, alcohol, and viral infection. The incidence of head and neck squamous cell carcinoma (HNSCC) is rising globally, particularly among the younger population, and is expected to reach 30% by 2030.[3]

Cancer metastasis is an important factor to consider when determining the treatment plan for SCC patients.[4] Over two decades, OSCC survival rates have been improved, but the overall prognosis remains unfavourable, with survival rates of about 47–74%.[5] Unfortunately, the presence of regional metastasis, which is the spreading of cancer cells to nearby anatomical regions in HNSCC, significantly decreases the survival rate by 50%.[6] Lymph nodes are the most common anatomical sites where metastatic HNSCC is found.[7] Nodal metastasis occurs when primary tumour cells infiltrate the lymphatic circulation and migrate to the regional lymph nodes in the neck, specifically cervical lymph nodes,[8] which is found in approximately 40% of all OSCC cases.[9]

Analyses of gene expression in both primary and metastatic HNSCCs have revealed the presence of numerous genes related to the process of cancer metastasis. These genes are involved in various biological processes, including epithelial-mesenchymal transition (EMT), stem cell characterisation, angiogenesis, lymphangiogenesis, extracellular matrix (ECM) degradation, resistance to cell death, and metabolic reprogramming. All of these biological events mainly contribute to regional and distant cancer metastasis.[10] Moreover, treatments for recurrence and

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metastasis of HNSCC are still challenging and require complicated medical approaches. There is a need for novel alternative agents to prevent or inhibit the metastasis of HNSCC. Thus, modulating and controlling metastasis-related genes is of interest to potentially regulate cancer severity and enable targeted treatment.

Matrix metalloproteinase (MMP) is a family of proteins that play various roles in ECM and basement membrane degradation. In HNSCC cells, several MMPs also play a significant role in the metastasis process due to their ability to promote cell proliferation, angiogenesis, and activate growth factors.[11] Interestingly, MMPs play a pivotal function in inducing the major alterations within the tumour microenvironment (TME) by enabling tumour cell infiltration and migration during the EMT process.[12] Moreover, the gene expression levels of several *MMPs* are higher in HNSCC cells compared to normal tissues, including such as *MMP1, MMP3, MMP7, MMP8, MMP9, MMP10, MMP11, MMP12, MMP13, MMP14,* and *MMP16*.[13]

Milk is a multifaceted biological liquid primarily composed of water, carbohydrates, lipids, proteins, and several bioactive compounds.[14] The composition of milk differs among species, while human and bovine milk share similar compositions, including protein, fat, and lactose. However, human milk has 9 g of protein, whereas bovine milk contains 34 g.[15] Alpha-lactalbumin (LA) and beta-lactoglobulin is the major protein that can be found in milk, accounting more than 50%.[16] LA is a protein found in both human and bovine milk, sharing 74% identical amino acid sequences. Human milk contains 36% LA, while bovine milk contains 20–25%. Several essential amino acids, including tryptophan, lysine, cysteine, leucine, and isoleucine, are found in LA.[17] Various techniques has been used to extract LA from milk, such as chromatography, membrane filtration, precipitation, aggregation, and enzymatic hydrolysis.[18] Interestingly, previous reports suggested that LA has promising activity in cancer cell modulation.[19] Human alpha-lactalbumin made lethal to tumours (HAMLET), comprises the combination of partially unfolded LA and oleic acid, which has demonstrated its potential as an anti-cancer agent.[19] The therapeutic effects of HAMLET were effective against the human glioblastoma xenograft model.[20] Several studies have shown that complex of HAMLET or bovine alpha-lactalbumin made lethal to tumours (BAMLET), and oleic acid exhibit cytotoxic activity against HNSCC cells. This cytotoxicity is hypothesised to occur through mechanisms such as cell cycle arrest, autophagy, and necrosis.[19]

Lactalbumin enzymatic hydrolysate (LAH) is the hydrolysis product of LA that carried out by applying a protein hydrolysis assay. The efficacy of enzymatic hydrolysis relies on the accessibility of peptide bonds, the stabilisation of protein structure, and the regulation processing time.[21] Enzymatic hydrolysis produces small peptides and amino acids, which enhancing the nutritive function without affecting nutritional value and improving health status, such as immunomodulatory, anti-cholesteremic, anti-thrombotic, anti-hypertensive, and anti-cancer activities.[22] However, the effects of LA or LAH on oral cancer cells remain unclear. In this study, we investigated the activity of bovine milk-derived LAH on HNSCC cell lines, *in vitro*.

2. Materials and methods

2.1. Cell lines

Human squamous cell carcinoma (hSCC) cell lines were used in this study, including tongue squamous cell carcinoma (HSC)-derived cell lines, HSC-4 (purchased from Japanese Collection of Research Bioresources Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition, Japan) and HSC-7 (gift from Dr. Teruo Amagasa, Tokyo Medical and Dental University, Japan) and head and neck (pharyngeal-derived) squamous cell carcinoma (HN) cell lines, HN-30 and HN-31 (gifts from Dr. J. Silvio Gutkind, University of California San Diego, USA). HSC-7 and HN-30 cells were collected from the primary tumour site, while HSC-4 and HN-31 were regional lymph node metastatic cells. Immortalised human periodontal ligament cell (i-hPDL) (purchased from Applied Biological Materials, Inc, British Columbia, Canada) was used for the control. The experiments and use of human cell lines were approved by the Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (approval no. HREC-DCU 2023–008).

2.2. Cell culture

Cells were cultured with the complete medium containing Dulbecco's Modified Eagle medium (DMEM, Thermo Fisher Scientific, USA), 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, USA), 2 mM L-glutamine (GlutaMAX™-I, Thermo Fisher Scientific USA), and 100 unit/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Antibiotic-antimycotic, Gibco, USA) in a humidified incubator with 5% CO_2 at 37 °C. Cells were passaged at 80–90% confluency, collected and used in all experiments.

2.3. Lactalbumin enzymatic hydrolysate (LAH) preparation

Lactalbumin enzymatic hydrolysate (LAH, Sigma Aldrich, Germany, L9010-500G) was dissolved in distilled water at a concentration of 100 mg/ml. LAH solution was further filtered with a single-use 0.2 μm filter unit (Sartorius Stedim Biotech, Germany) and kept in a −20 °C freezer before use.

2.4. Cytotoxicity assay

hSCC and i-hPDL cells (density 1.5×10^4 cells/well) were seeded in the 24-well plates (Thermo Fisher Scientific, USA) in complete medium and incubated overnight. Cultures were treated with a 2-fold serial dilution of LAH (range of concentration 0.39–100 mg/ml) in a 5% CO_2 humidified incubator at 37 °C for 24 h. Next, cells were incubated in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (Tocris, UK) for 30 min, washed with phosphate buffer saline (PBS), and dissolved using glycine buffer in dimethyl sulfoxide (DMSO, Sigma Aldrich, Germany) for 30 min. A microplate reader (Molecular Devices, USA) was used to measure the absorbance at wavelength 570 nm (OD_{570}).

2.5. Proliferation assay

hSCC cells (density 3.5×10^3 cells/well) were seeded in the 96-well plates (Thermo Fisher Scientific, USA) and treated with LAH at a two-fold serial concentration from 0.39 to 12.5 mg/ml. Cultures were incubated in a humidified incubator with 5% CO_2 at 37 °C for 1, 3 and 5 days. Cells were then incubated in MTT solution, as previously mentioned, and the optical density at 570 nm was measured by a microplate reader.

2.6. Colony-forming unit (CFU) assay

hSCC cells (density 1×10^3 cells/well) were seeded in the 24-well plates and maintained with complete medium in a humidified incubator with 5% CO_2 at 37 °C overnight. Cultures were treated with LAH at concentration 6.25–12.5 mg/ml for 5 or 12 days. Cells were fixed in 4% formalin in PBS for 10 min, washed with PBS. The attached cells were stained with 0.1% crystal violet blue (Sigma Aldrich, Germany) and incubated at room temperature for 10 min. Next, stained cells were washed with PBS and dried before observation by an inverted microscope (Nikon T2S, Japan). For quantitative analysis, stained cells were dissolved in glycine buffer with DMSO and shaken using a microplate shaker for 90 min. The eluted solution was transferred to the 96-well-plates. The absorbance was measured using a microplate reader at the optical density 570 nm (OD_{570}). The relative absorbance value of LAH treatment group was normalized to control group.

2.7. RNA isolation and reverse transcriptase real-time polymerase chain reaction (RT-qPCR)

hSCC cells (density 1 × 10^5 cells/well) were seeded in the 12-well plates (Thermo Fisher Scientific, USA) and maintained with complete medium in a humidified incubator with 5% CO_2 at 37 °C overnight. Then, cultures were treated with 6.25 mg/ml LAH for 24 h.

Cells were homogenised with 1 ml of Trizol reagent (RiboEx™ total solution, GeneAll, South Korea) and total RNA was extracted according to the manufacturer's protocol. The final concentration of RNA was measured by the Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA). Total RNA (1 μg) was converted to complementary DNA (cDNA) by CFX Connect Real-Time PCR (Bio-Rad, USA) following manufacturer's instruction.

Reverse transcriptase real-time PCR (RT-qPCR) was performed by using SYBR Green Reaction Mix (Roche Diagnostic, Germany) and mRNA expression was assessed by the MiniOpticon real-time PCR system (Bio-Rad, USA). Melting curve analysis was performed and relative mRNA expression was normalized with *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene and control group by calculated by $2^{-\Delta\Delta CT}$ method.[23] Oligonucleotide sequences used for RT-qPCR were shown in Supplementary Table 1.

2.8. Migration assay

A scratch wound healing assay was performed. hSCC cells (density 6 × 10^5 cells/well) were seeded in the 6-well plates (Thermo Fisher Scientific, USA) and maintained with complete medium in the humidified incubator overnight. Then, cultures were washed with PBS and incubated with FBS-free DMEM medium for 24 h. Vertical wound healing was created by a 1000-μl pipet tip (Axygen T-1000-B, Corning, USA), washed detached cells and cell debris with PBS and treated with FBS-free DMEM medium containing LAH at concentration 0.781–6.25 mg/ml. Scratch wound closure was observed, and the images were captured by an inverted microscope (Nikon TS2, Japan) at 0, 24, and 48 h, respectively. The area of the scratch wound was measured using ImageJ software (National Institute of Health, USA). The percentage of wound closure was calculated by the following formula.

$$\text{The percentage (\%) of wound closure} = (T_N/T_0) \times 100$$

T_0 = Initial wound area (0h)
T_N = Remaining wound area at 24 or 48h

2.9. Statistical analysis

All experiments were conducted in triplicate at least three times. The data were shown as mean and standard deviation (S.D.). Statistical analysis was calculated by Prism 9 (GraphPad Software, USA). An independent student's t-test (two-tailed) was used for comparison between the two groups. One-way ANOVA followed by Dunnet's post-hoc test was performed for multiple comparisons. *P value* < 0.05 was considered as statistical significance.

3. Results

3.1. Cytotoxicity of LAH

To investigate the activity of LAH on human squamous cell carcinoma (hSCC) cell lines *in vitro*, we initially investigated the cytotoxicity of LAH by an MTT assay. hSCC cell lines, including HN-30, HN-31, HSC-7 and HSC-4, were treated with a two-fold serial dilution of LAH

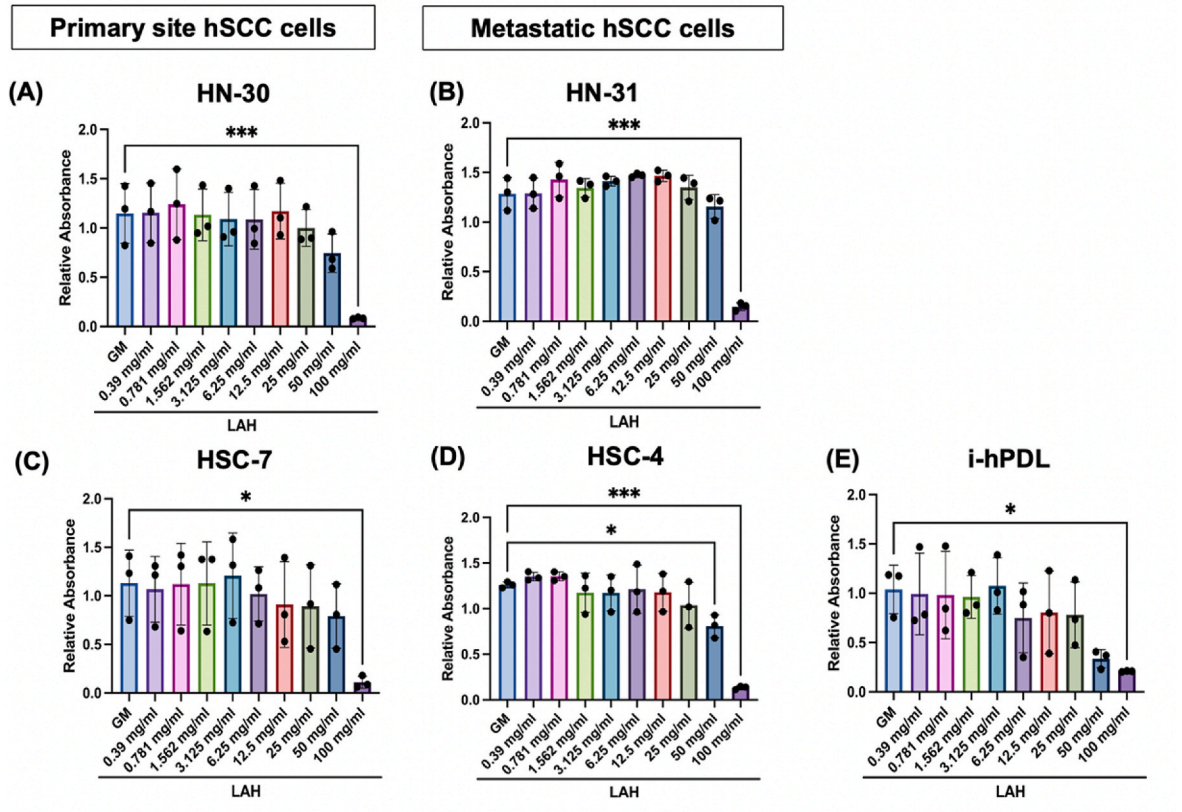

Fig. 1. Cytotoxicity of LAH on hSCC cells Cytotoxicity of LAH on hSCC and i-hPDL cells was examined by an MTT assay. Cells were treated with a two-fold of LAH (0.39–100 mg/ml) for 24 h. **(A–B)** Cell viability assay of HN-30 and HN-31, **(C–D)** HSC-7 and HSC-4 and **(E)** i-hPDL cells. Statistical analysis was performed by One-way ANOVA followed by Dunnet's post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(range 0.39–100 mg/ml) for 24 h. LAH at 100 ng/ml significantly decreased the cell viability of all hSCC cell lines after 24 h (Fig. 1A–D). In addition, 50 mg/ml of LAH significantly reduced cell viability of HSC-4 cells (Fig. 1D). In the normal human cell control, immortalised human periodontal ligament cells (i-hPDL) treated with 50 and 100 mg/ml LAH also showed decreased cell viability (Fig. 1E). Therefore, LAH at a concentration of 0.39–12.5 mg/ml had no cytotoxicity on both hSCC and normal human cells. The concentrations of LAH mentioned were used in all subsequent experiments.

3.2. LAH reduced cell proliferation of hSCC cell lines

The proliferation of hSCC cell lines was investigated on day 1, 3 and 5 after cultured with 0.39–12.5 mg/ml LAH using an MTT assay. On day 5, the proliferation of HN-30 cells was reduced when treated with 1.5625–12.5 mg/ml LAH, while HN-31 cells exhibited significantly attenuated cell proliferation with 6.25 and 12.5 mg/ml LAH compared with the control (Fig. 2A and B). Further, HSC-7 cells treated with 1.5625–12.5 mg/ml LAH also showed a significant reduction in proliferation on day 5 (Fig. 2C) and LAH-treated HSC-4 cultures revealed the same tendency of attenuated proliferation (Fig. 2D). In particular hSCC cell lines, higher doses of LAH reduced cell proliferation on day 3, including HN-31, HSC-7 and HSC-4. Thus, LAH at concentrations of 6.25 and 12.5 mg/ml has the ability to attenuate the proliferation of hSCC cell lines.

To confirm the LAH activity on hSCC cell proliferation capability, we performed the colony-forming unit (CFU) assay. hSCC cell lines were treated with 6.25 and 12.5 mg/ml LAH for 5 or 14 days. The cells cultured in complete medium without LAH were used as the control. The results demonstrated that LAH at a concentration of 12.5 mg/ml significantly inhibited colony formation of all tested hSCC cell lines (Fig. 3A–D). HN-30 cells also significantly reduced the colony formation when treated with 6.25 mg/ml (Fig. 3A). The reduction in both colony number, size, and cell density was markedly observed. Therefore, the findings indicated that LAH has the potential to control hSCC cell proliferation and colony formation ability in both primary site and regional metastatic hSCC cell lines *in vitro*.

3.3. LAH attenuated MMP expression on hSCC cell lines

Matrix metalloproteinases (MMP) belong to the protease enzyme family, the calcium-dependent zinc-containing endopeptidase.[24] Importantly, MMPs play a pivotal role in tumour progression by facilitating the degradation of basement membrane and then promoting the process of cancer metastasis.[11] Dysregulation of multiple MMPs, such as MMP1, MMP2, MMP7, MMP9, MMP13, and MMP14, is frequently observed in several human cancers.[25]

To investigate the activity of LAH on metastasis-related *MMP*s transcription level in hSCC cell lines, the expression of *MMP1*, *MMP2*, *MMP9*, and *MMP14* was assessed by the RT-qPCR analysis. hSCC cell lines were treated with 6.25 mg/ml LAH for 24 h hSCC cells cultured in a complete medium without LAH were used for the control group. In hSCC cell lines that were isolated from the primary tumour site, the mRNA expression of *MMP1, MMP2, MMP9* and *MMP14* was significantly reduced in HN-30 cells, while in HSC-7 cells, only the mRNA levels of *MMP2* and *MMP14* were downregulated (Fig. 4A and C). In the metastatic hSCC cell line group, the expression of *MMP2*, *MMP9*, and *MMP14* was significantly downregulated, while the gene expression of *MMP1* showed no significant difference compared to the control group (Fig. 4B). Moreover, only *MMP1* expression was significantly reduced in HSC-4 cells, a metastatic tongue hSCC. (Fig. 4D). Thus, the results suggest that LAH may exert partial control over the expression of *MMP* genes, which play a key role in the metastasis process of hSCC cells.

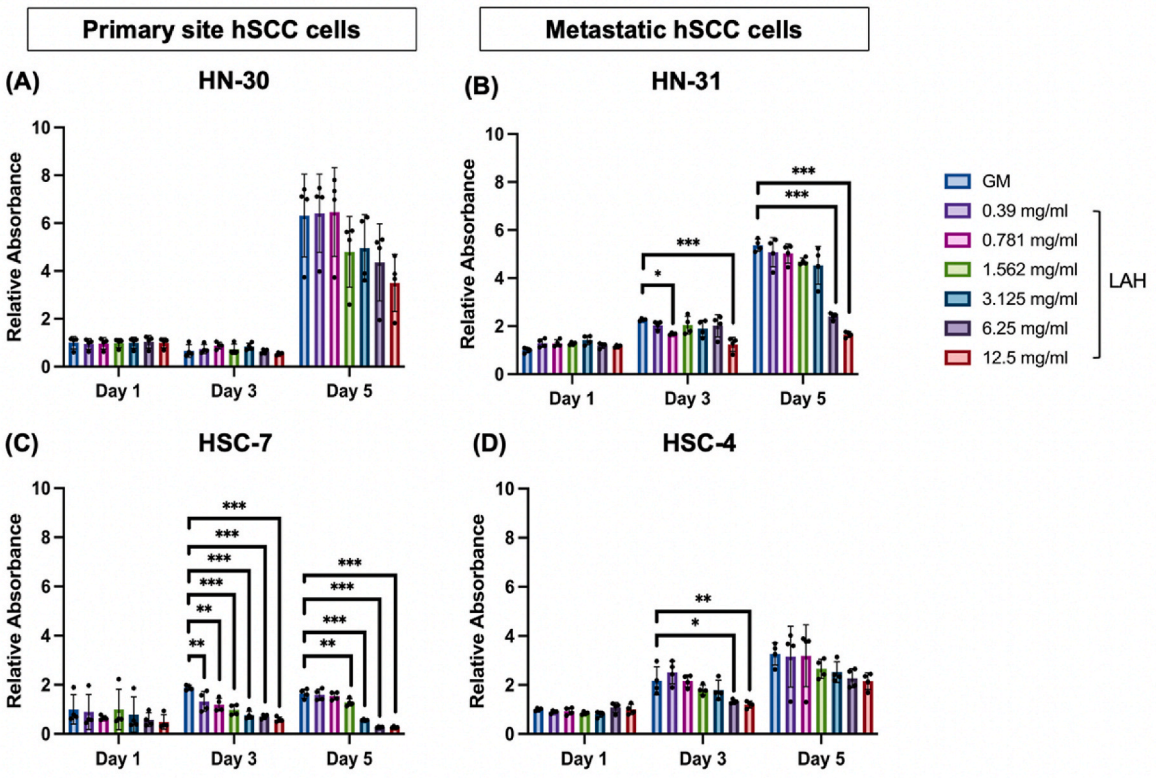

Fig. 2. LAH inhibited cell proliferation of hSCC cells hSCC cells were treated with 0.39–12.5 mg/ml of LAH for 5 days and examined by an MTT assay. **(A–D)** Cell proliferation of **(A)** HN-30, **(B)** HN-31, **(C)** HSC-7 and **(D)** HSC-4. Statistical analysis was performed by One-way ANOVA followed by Dunnet's post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

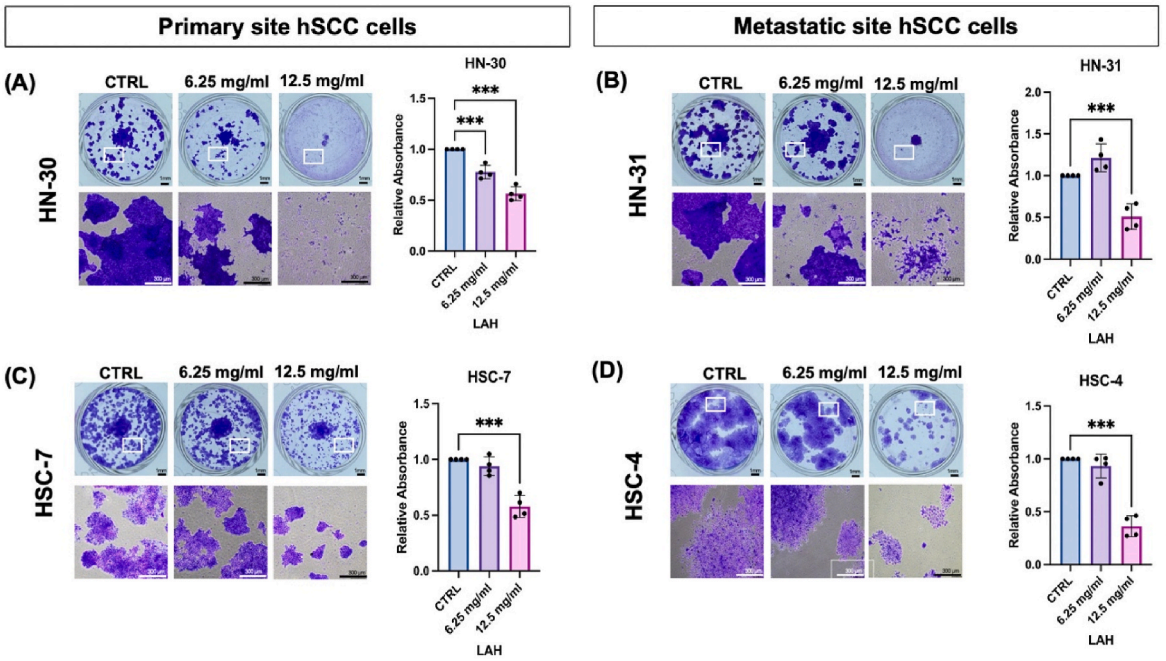

Fig. 3. LAH reduced colony formation ability of hSCC cells hSCC cells were treated with 6.25 and 12.5 mg/ml LAH. (A–D) The colony-forming unit (CFU) was assessed by a crystal violet blue staining. CFU representative images and quantitative analysis at OD570 of (A) HN-30, (B) HN-31, (C) HSC-7 and (D) HSC-4 after LAH treatment were shown. White rectangles indicate the ROI for higher magnification. Statistical analysis was performed by One-way ANOVA followed by Dunnet's post-hoc test. ***p < 0.001. Scale bar = 1 mm, 300 μm.

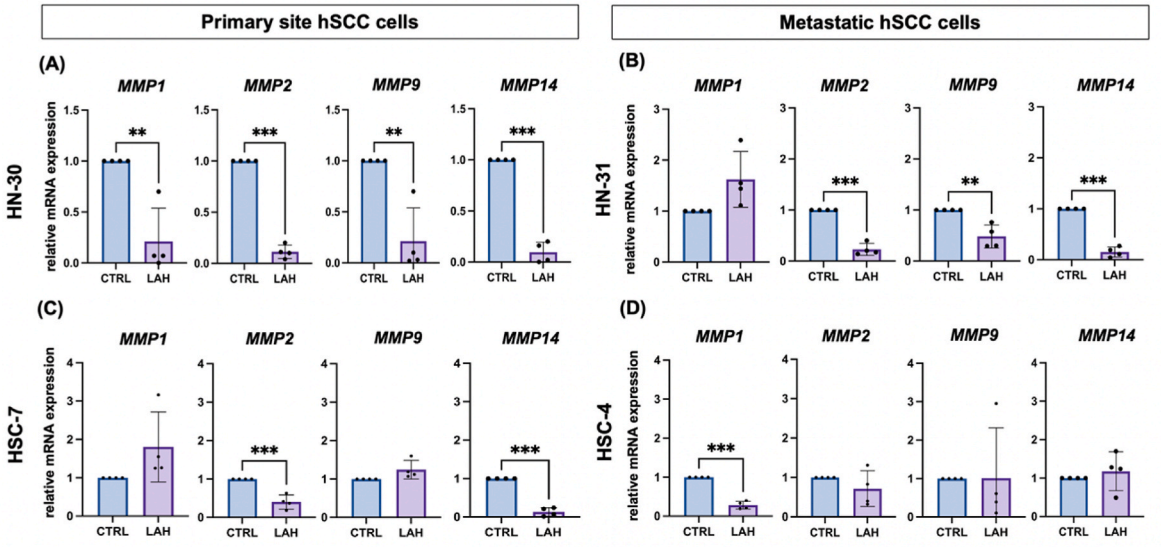

Fig. 4. LAH regulated *MMP* gene expression in hSCC cells **(A-D)** The mRNA expression of *MMP1*, *MMP2*, *MMP9* and *MMP14* in hSCC cells after 6.25 mg/ml of LAH treatment for 24 h by RT-qPCR. Relative mRNA expression of *MMPs* in **(A)** HN-30, **(B)** HN-31, **(C)** HSC-7 and **(D)** HSC-4. Statistical analysis was performed by independent student's t-test. ***p < 0.01; ***p<0.001.*

3.4. LAH did not affect hSCC cell migration

Since LAH attenuated *MMPs* expression, we next investigated the activity of LAH on cell migration of hSCC by using the scratch wound healing assay. hSCC cell lines were treated with 0.781–6.25 mg/ml LAH in FBS-free culture medium for 48 h. The percentage of wound healing area in the LAH-treated primary site-derived hSCC cells, HN-30 and HSC-7, was comparable to the control group (Fig. 5A–C). Similarly, lymph node metastatic-derived hSCC cells, HN-31 and HSC-4, did not exhibit different wound closure rates, even though LAH-treated HSC-4 cells showed higher wound healing percentages (Fig. 5 B-D). Therefore, these findings suggested that LAH does not have effect on the migration of hSCC cells *in vitro*.

4. Discussion

Controlling the metastasis of cancer is one of the current approaches among several cancer therapies. Because hSCC is one of the most prevalent types of head and neck cancers, and regional metastasis to nearby lymph nodes is a critical event in determining the severity of tumour,[1]

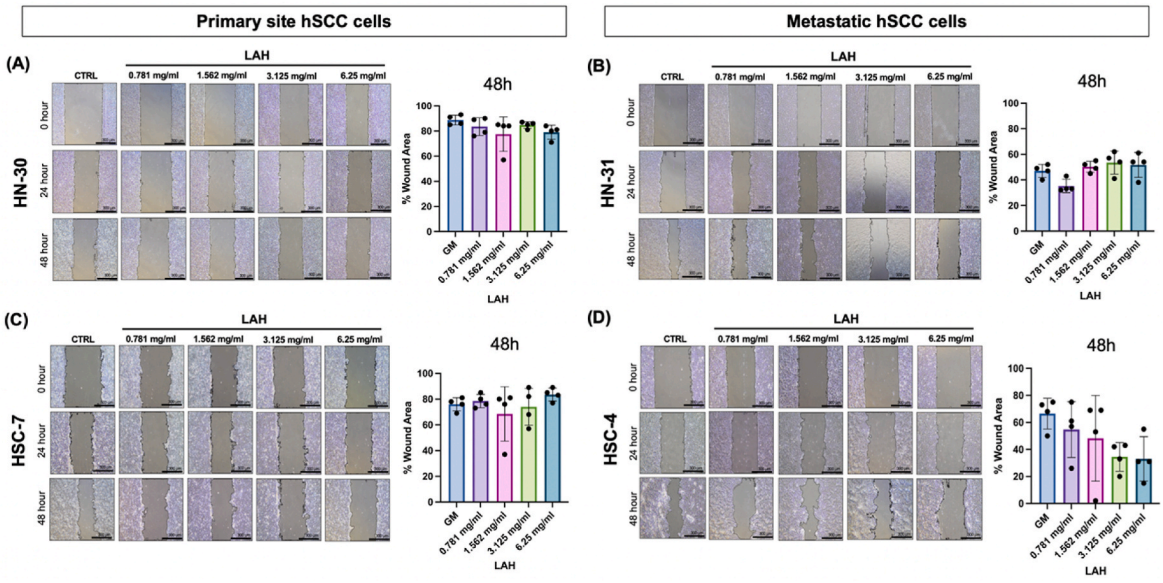

Fig. 5. LAH did not affect hSCC cell migration The scratch wound healing assay was performed for cell migration analysis of hSCC cells after LAH treatment for 48 h. **(A–D)** Representative images of cell migration at 0 h, 24h and 48 h. Percentage of wound healing area at 48 h was shown in bar graphs, **(A)** HN-30, **(B)** HN-31, **(C)** HSC-7 and **(D)** HSC-4. Statistical analysis was performed by One-way ANOVA followed by Dunnet's post-hoc test. Scale bar = 300 μM.

an alternative agent with anti-cancer or anti-metastasis properties is required to treat early stage tumour or premalignant lesions. The present study focused on the activity of a natural product derived from bovine milk, LAH, which possesses interesting properties against cancer cells and can be easily manipulated in a pre-clinical study.

LA is recognised as a significant source of essential amino acids, which include bioactive peptides concealed within their amino acid sequences.[26] Various techniques can be used to isolate LA from milk whey to achieve their various bioactivities such as anti-cancer, antimicrobial, antiviral, antihypertensive, and antioxidant properties.[27] Thus, we hypothesised that LAH, the hydrolysate product of LA should have potential biological functions against oral cancer cells, particularly hSCC cells. The findings indicated that LAH affects cell proliferation and controls metastasis-associated *MMP* gene expression without altering cell migration ability *in vitro*.

Uncontrolled and abnormal cell proliferation of tumour significantly impact cell cycle activity, initiation, and progression of cancer cells.[28] Our results indicated that LAH inhibited cell proliferation and CFU ability of hSCC cells in a dose-dependent manner, suggesting its potential as an effective anti-cancer agent at tested biocompatible doses. Similar to our finding, LA has been found to regulate cell proliferation in various cancers by inhibiting cell growth and controlling cell apoptosis.[29,30] The combination of LA and oleic acid exhibited cytotoxic activity against OSCC cells. Moreover, LA reduced cell proliferation and induced cell apoptosis in a human glioblastoma cells.[19,20] Of note, the anti-proliferative activity of LA and its enzymatic hydrolysate derivatives is mainly related to the apoptosis of tumour cells. Therefore, further studies are needed to investigate the mechanism of LAH on hSCC cell proliferation in order to confirm whether it is related to cell cycle arrest, programme cell death or induction of cell necrosis.

Inhibiting the cancer metastasis process is a key strategy to prevent the progression of hSCC to local lymph nodes of the head and neck region. MMPs significantly contribute to the onset and progression of cancer, facilitating cell proliferation, invasion and metastasis through their proteolytic activity within the TME.[31] The abundance of MMPs expression in particular cancers, such as colon, breast, and ovary cancers, leads to their aggressiveness and poor prognosis.[32] Accordingly, in OSCC patients, the level of MMP in the blood, tissues, and saliva has the potential to be utilised as oral cancer biomarkers, including early identification, risk assessment, prognostic analysis, and evaluation of therapy response.[33] Thus, we decided to examine the expression of *MMP* genes that are highly associated with the metastasis process of hSCC cells, including *MMP1, MMP2, MMP9,* and *MMP14*, after LAH treatment.

We observed both upregulation and downregulation of mRNA expression levels of *MMP1*, *MMP2*, *MMP9*, and *MMP14* in different hSCC cells derived from various anatomical sites. In HN cells, we found the downregulation of *MMP1*, *MMP2*, *MMP9*, and *MMP14* in both primary and metastatic cancer cell lines after treated with LAH. However, in tongue hSCC cells (HSC cell), the inhibition of *MMP* transcripts appeared to be more effective in a primary site cancer cells compared to the lymph node metastatic cells. The MMP1 overexpression in HNSCC tissues promotes cell metastasis, affecting treatment outcomes and prognosis.[34] Similarly, the overexpression of MMP1 in OSCC influenced the bone metastasis event due to its role in degradation of collagenous matrix components.[35] The results also indicated the reduction of *MMP2* mRNA level after exposed to LAH. MMP2 is the important MMP protein and marked as a tumour maker. The expression of MMP2 is often associated with tumour growth and invasion of cancer and metastatic capabilities of oral SCC.[36,37] Thus, downregulation of *MMP2* transcripts by LAH may contribute to the decrease of tumour aggressiveness to some extent. The role of MMP9 in promoting invasion has been established, and its association with an unfavourable prognosis has been observed in HNSCC patients.[37] It has been known that MMP9 has the capability to break down ECM, an important structural component within the TME. The process of cancer progression involves substantial remodeling of this tissue component.[38] Indeed, MMP14 has been identified as a potential prognostic indicator for metastasis in oral SCC, particularly in cases when lymph nodes are affected. The association between MMP14 and the development of oral SCC can be defined by its intrinsic proteolytic activity, as well as its capacity to activate other MMPs overexpression that may have detrimental effects.[33]

Of note, LAH treatment appeared to regulate *MMP* expression more effectively in primary site-derived than in metastatic-derived hSCC cells. This finding indicated that the activity of LAH varies among different hSCC cell lines, possibly due to underlying cellular responses and cell behaviour that require further investigation. In addition, the mRNA and protein expression of those MMPs should be confirmed in other types of hSCC to validate and broadening LAH activity for further pre-clinical studies.

Cancer cell migration and invasion though blood or lymph vessels

are crucial in tumour metastasis, facilitated by downregulation of E-cadherin and upregulation of proteins such as mesenchymal vimentin and N-cadherin.[39,40] We also assessed the LAH activity on cell migration using a scratch wound healing assay; however, the tested concentrations of LAH had no effect to inhibit cell migration in either primary or metastatic hSCC cells within 48 h. This may be due to the concentration of LAH or the limitation of scratch wound healing assay.

Our present study suggested the preliminary data regarding *in vitro* LAH activity on hSCC cell lines can serve as new knowledge for further application and expansion in other pre-clinical and animal studies since enzymatic hydrolysis of dietary protein produces several small peptides that enhancing their functions such as anti-cancer activity.[41] Anti-cancer peptides (ACPs) are suggested as a promising anti-cancer agent with the potential to surpass conventional approaches. ACPs interact with cancer cells, resulting in cell necrosis, cell apoptosis, suppression of angiogenesis, and activation of immune cells.[42] The hydrolysed form of BAMLET induced the anti-cancer activity by inhibiting the cell proliferation, reducing cell viability and enhancing cytotoxicity effects in tumours. Conversely, it appeared to promote the cell viability and proliferation in normal cells.[43] Development of LAH with other active molecules could be the promising approach to investigate its activity on hSCC as previously reported. For example, HAMLET, LA-oleic acid complex, has targeted cytotoxic effects on cancer cells by initiating apoptosis process while leaving normal cells unharmed. This indicates that HAMLET has the ability to bypass various apoptosis resistance mechanisms that are present in cancer cells.[44] The systemic administration of a human LA complex resulted in a reduction in both tumour size and the number of polyps in the murine colon cancer model.[45] Therefore, combining LAH with a lipid complex should be a better strategy for the drug delivery system in hSCC therapy. Another promising strategy is the combination of LAH and drug vehicle, such as hydrogel, as a topical therapy for treatment small OSCC and premalignant lesions. However, there have been limited studies on LAH and its effects on cancer cells, both *in vitro* and *in vivo*. Further research on bovine milk-derived LAH production, formula, manufacturing, and its activity in animal models are required to optimize biocompatibility and determine the safest dose of LAH for the living body.

Taken together, we assessed the effects of LAH on the proliferation, gene expression and migration of hSCC cell lines. *In vitro* studies may not accurately represent the complex conditions and environment of the human body, such as cancer development and progression in TME, due to their controlled and simplified environment. Further mechanistic examination is required to elucidate the beneficial effects of LAH as an adjuvant treatment in oral squamous cell carcinoma.

5. Conclusion

LAH has inhibitory effects on the proliferation and growth of the primary and metastatic cells from the tongue and pharyngeal SCC. Furthermore, LAH downregulated the mRNA expression of *MMP1, MMP2, MMP9,* and *MMP14*. These results indicated that LAH has potential effects as a therapeutic agent for HNSCC. However, an *in vivo* study is indeed necessitated to investigate the involved pathway and the potential clinical implication of LAH on HNSCC treatment.

Author Contributions

AA and WN designed and performed the projects. AA, WN, and PL interpreted the results. TO contributed to provide the material and methods during the experiment. WN and TO contributed to the conceptual design and supervised during experiments. AA wrote the first draft of the manuscript AA, TO and WN critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Ethics approval statement

The protocol was conducted in accordance with the Declaration of Helsinki and approved by the Human Research Ethical Committee of the Faculty of Dentistry, Chulalongkorn University (approval No. HREC-DCU 2023-008).

Declaration of competing interest

The authors declare no competing interests.

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List of Abbreviations

ACP	Anti-cancer peptide
BAMLET	Bovine alpha lactalbumin made lethal to tumour
CFU	Colony-forming unit
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HAMLET	Human alpha lactalbumin made lethal to tumour
hSCC	Human squamous cell carcinoma
HNSCC	Head and neck squamous cell carcinoma
i-hPDL	Immortalised human periodontal ligament cell
LA	Alpha Lactalbumin
LAH	Lactalbumin enzymatic hydrolysate
MMP	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
OSCC	Oral squamous cell carcinoma
SCC	Squamous cell carcinoma
TME	Tumour microenvironment

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jobcr.2024.02.011.

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